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Resistin down-regulates insulin receptor expression, and modulates cell viability in rodent pancreatic beta-cells

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Abstract **The adipokine resistin is known to induce insulin resistance in rodent tissues. Increases in adipose tissue mass are known to have a negative effect on pancreatic beta-cell function, although the mechanisms are poorly understood. This study investigated the effects of resistin on insulin secretion, insulin receptor expression and cell viability in pancreatic beta-cells. BTC-6 or BRIN-BD11 cells were treated for 24 h with resistin, and insulin receptor expression, insulin secretion and cell viability were measured. Incubation with 40 ng/ml resistin caused significant decreases in insulin receptor mRNA and protein expression, but did not affect insulin secretion. At low concentrations, resistin caused significant increases in cell viability. These data implicate resistin as a factor that may regulate beta-cell function/viability, and suggests a potential mechanism by which increased adiposity causes beta-cell dysfunction.**
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1. Introduction

It is well recognised that together with reduced insulin sensitivity, impaired pancreatic islet beta-cell function is one of the two key components in the pathogenesis of type 2 diabetes (T2D), a condition which is expected to affect over 300 million people worldwide by 2025 [1]. The precise mechanisms by which this loss of islet beta-cell function occurs are however poorly understood. One factor often overlooked is the role of insulin receptor in the beta-cell, as not only are insulin receptors expressed in the beta-cell [2], they have been shown to play a role in regulating beta-cell function, including insulin secretion [3] and beta-cell mass [4].

Although T2D has a multi-factorial aetiology, one very well-established risk factor for T2D is obesity [5], with over 90% of sufferers of T2D in Western societies being obese. Until recently adipose tissue was thought to act primarily as an energy reservoir, allowing the body to negotiate times of famine by storing triacylglycerol in times of excess, and mobilising it in times of shortage. A large volume of recent research has however identified adipose tissue as a metabolically active endocrine organ that is able to secrete a significant number of bioactive peptides that have been termed 'adipokines'. These molecules include hormones, growth factors and cytokines such as leptin, resistin, visfatin, adiponectin and TNF-α [6]. It has also been shown that these secretory molecules regulate a wide variety of cellular functions relevant to both the pathogenesis and complications of diabetes.

Resistin, a 12.5 kDa cysteine rich adipokine, has been identified as a potential link between obesity and diabetes [7,8]. Initial research showed that resistin induced insulin resistance in mice when injected into normal mice, and that treating diet-induced diabetic mice with anti-resistin antibody reduced blood glucose and improved insulin action [5]. Although this initial finding has been repeated in rodents, the ability of resistin to induce insulin resistance in human tissue is much more controversial [9,10]. Resistin expression in adipocytes has also been shown to be regulated by a wide range of factors including insulin [11], tumour necrosis factor-alpha [12], metformin [13] and oestrogen [14].

Interestingly, recent evidence has demonstrated that resistin is not expressed exclusively in adipose tissue, with at least one group demonstrating its expression in islet tissue and clonal beta-cells [15]. Resistin expression has also been identified in the liver [16] and it has been suggested that it is rather in liver disease that resistin may induce insulin resistance in humans [17]. In lean subjects, the serum levels of resistin have been reported to be approximately 5–15 ng/ml [18,19], whereas in either T2D or obesity, the serum level increases to as high as 40 ng/ml [20]. These observations reinforce the theory that in the obese and/or diabetic condition, islet tissues are exposed to higher than normal concentrations of resistin.

The expression of resistin in islet tissue could possibly suggest an islet or beta-cell specific role for resistin, however there is little published research into any potential role for resistin in regulating beta-cell function. This study therefore aimed to investigate the effect that pathological concentrations of resistin had on pancreatic beta-cell function, viability and insulin receptor gene expression.

2. Materials and methods

2.1. Cell culture and treatment

All materials were purchased from Sigma (Poole, UK) unless otherwise stated.

βTC-6 cells were purchased from the ATCC collection (LGC Promochem, UK), BRIN-BD11 cells were a gift from Prof. Peter Flatt (University of Ulster, Coleraine, Northern Ireland, UK). Cells from

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both cell lines between passages 30–45 were cultured in standard 11 mmol/l glucose RPMI-1640 (supplemented with 2 mmol/l L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin, 10% foetal bovine serum) in a 5% CO_2 incubator at 37 °C incubator. All treatments were performed in supplemented but serum-free RPMI-1640.

For experimental treatments, cells were seeded into T75 flasks (βTC-6 cells for mRNA and protein expression), 1.5×10^5 cells per well in a 24 well plate (BRIN-BD11 cells for insulin secretion) or 0.5×10^4 in 96-well plates (βTC-6 cells for modified MTS cell viability assay). Cells were allowed to attach for 24 h and subsequently were incubated with recombinant rat resistin (Cambridge Biosciences, Cambridge, UK) for 24 h. For insulin receptor expression and insulin secretion assays a concentration of 40 ng/ml resistin was used, for cell viability a range of 0–40 ng/ml was used.

2.2. Measurement of insulin receptor mRNA and protein expression

For mRNA expression experiments, total RNA was extracted using an SV Total RNA Isolation kit (Promega, Southampton, UK) and was quantified and assessed using gel electrophoresis. One microgram of total RNA was reverse transcribed using AMV reverse transcriptase (Promega, Southampton, UK) and oligo dT_{15} primers (Bioline, UK).

For real-time PCR, resulting cDNA's were diluted 1 in 2 and subsequently amplified for 40 cycles in an iCycler (Biorad, Hemel Hempstead, UK) using a proprietary SYBR Green Fluorescein Mix (Primerdesign, Southampton, UK) and in house designed sequence specific primers for mouse insulin receptor (sense primer AATGGCAACATCACACACTACC; anti-sense primer CAGCCCTTTGAGACA-ATAATCC). For housekeeping genes and resistin expression, pre-validated mouse actin, GAPDH and resistin primers were purchased (Primerdesign, Southampton, UK). Relative expression of insulin receptor to housekeeping genes was calculated by comparison of C_t values (delta − delta C_t). Primer efficiencies for both genes of interest and housekeeping genes were between 95% and 100% as measured by Linreg software (supplied by Primerdesign, Southampton, UK).

For protein expression experiments, total cellular protein was extracted from control/treated cells for 1 h at 4 °C in a detergent based cell lysis buffer (20 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X, protease inhibitor cocktail) before being quantified using DC protein assay kit (Biorad, Hemel Hempstead, UK). Cell lysates were then centrifuged at 13000 G for 10 min to separate out protein and boiled. 40 μg total protein was loaded per sample and run through a 4–8% polyacrylamide gel. Samples were subsequently transferred to a nitrocellulose membrane (Geneflow, Lichfield, UK), blocked using 5% non-fat milk and probed using rabbit polyclonal anti-insulin receptor (Autogen Bioclear, UK) at 1:300 at 4 °C overnight and rabbit anti-goat IgG-conjugated secondary antibody (Autogen Bioclear, UK) at 1:2000 at room temperature for 1 h. After a series of washes in TBS-T, membranes were developed using ECL+ detection kit (Amersham, Little Chalfont, UK) and images were visualised using a Storm® Phosphorimager (Amersham, Little Chalfont, UK). Band density was measured using Scion image software (NIH). To ensure equal band loading, samples were also probed for beta-actin protein expression using a polyclonal rabbit anti-beta actin antibody at 1:500 dilution (Autogen Bioclear, UK).

2.3. Cell viability

For measurement of cell viability, cells were plated out at a density of 2×10^4 cells per well in a 96-well plate and were treated as described above for 24 h. After this period, CellTiter 96® Aqueous One Solution Cell Proliferation Assay solution (Promega, Southampton, UK) was added for 2 h. Absorbance at 490 nm was measured and recorded.

2.4. Insulin secretion

For insulin secretion, the reportedly glucose responsive clonal rodent pancreatic beta-cell line BRIN-BD11 was used. BRIN-BD11 cells between passages 30 and 36 were plated out at a density of 0.2×10^4 cells per well in a 96-well plate and allowed to attach overnight. Cells were subsequently treated with 0, 20 and 40 ng/ml resistin for 24 h in serum free RPMI. After this period, cells were immersed in RPMI containing 2.2 mm glucose and then stimulated with 22 mM glucose, the media from this last treatment being collected and assayed for insulin content using a proprietary Rat insulin ELISA (Mercodia, Sweden).

2.5. Statistical analysis

Statistical analysis of all data obtained was undertaken using the SPSS v.10 statistical analysis package. For all analysis, normal distribution was assumed due to analysis of similar previous experimentation using these cells. For single comparisons (control vs treatment) a two tailed unpaired *t*-test was employed; where multiple comparisons were required ANOVA with Tukey's post-hoc test was applied. $P \leqslant 0.05$ was taken as the point of significance. For each set of experiments, where '*n*=' is stated, '*n*=' refers to individual experiments, each run in either quadruplicate or quintuplicate. All graphs are displayed as means ± S.E.M. * denotes $P < 0.05$.

3. Results

3.1. Resistin reduces insulin receptor mRNA expression

Qualitative real-time SYBR Green® evaluation of insulin receptor mRNA expression showed that when βTC-6 cells were incubated with 40 ng/ml resistin for 24 h, a significant decrease of 70% (Fig. 1) compared to control was seen ($P = 0.0005$).

3.2. Resistin reduces insulin receptor protein expression

Twenty-four-hours incubation with 40 ng/ml resistin also caused a marked reduction in the level of insulin receptor protein expression detectable by Western blot. Resistin treated samples gave a detected band intensity of approximately 60% less than control samples (Fig. 2).

3.3. Resistin increases cell viability at physiological, but not pathological concentrations

Twenty-four-hours incubation with resistin caused significant increases in cell viability as assessed by modified MTS assay compared to control when βTC-6 cells were incubated at

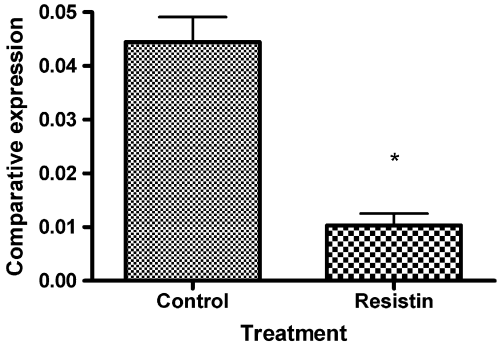

Fig. 1. Resistin decreases insulin receptor mRNA expression. Incubation with 40 ng/ml resistin for 24 h caused a significant 70% decrease in the levels of insulin receptor mRNA compared to control as detected by real-time PCR ($P = 0.0005$). $n = 4$.

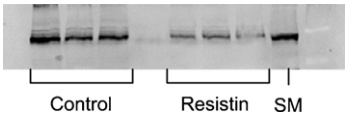

Fig. 2. Resistin decreases insulin receptor protein expression. Twenty-four-hours incubation with 40 ng/ml resistin also caused a marked decrease in the levels of insulin receptor protein compared to control, with a decrease in detected band intensity of 60% being recorded. Mouse skeletal muscle cell lysate (SM) was used as a positive control ($n = 3$ for each treatment).

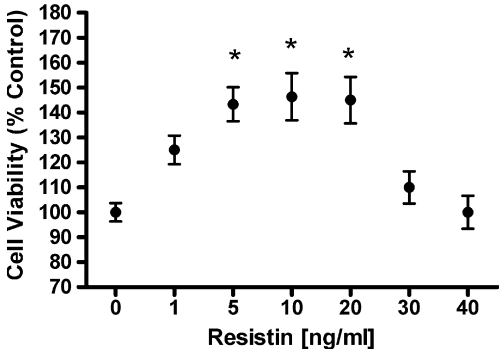

Fig. 3. Resistin increases βTC-6 cell viability at normal but not elevated levels. Incubation with concentrations from 5 to 20 ng/ml resistin caused significant increases of as much as 45% in cell viability compared to control ($P < 0.01$). However, when the concentration of resistin was increased to 30–40 ng/ml these increases in cell viability were not seen ($n = 5$ for each treatment).

lower and more 'physiological' concentrations (10–20 ng/ml), but at higher 'pathological' concentrations associated with obesity and diabetes (30–40 ng/ml), these increases in cell viability were not seen (Fig. 3), resulting in a bell-shaped response curve. Similar results were seen using BRIN-BD11 cells.

3.4. Resistin mRNA is expressed in βTC-6 and BRIN-BD11 cells

Using both standard and real-time PCR to evaluate resistin mRNA expression in βTC-6 and BRIN-BD11 cells, resistin mRNA was detected in both cell lines (data not shown). Using real-time PCR the cycle threshold (C_t) seen in these cells was between 23 and 26, suggesting a relatively high level of expression.

4. Resistin has no effect on insulin secretion from BRIN-BD11 cells

Static incubation for 24 h with 40 ng/ml resistin had no effect on high glucose (22 mmol/l) stimulated insulin secretion over 1 h from the reportedly glucose responsive BRIN-BD11 cell line (Fig. 4).

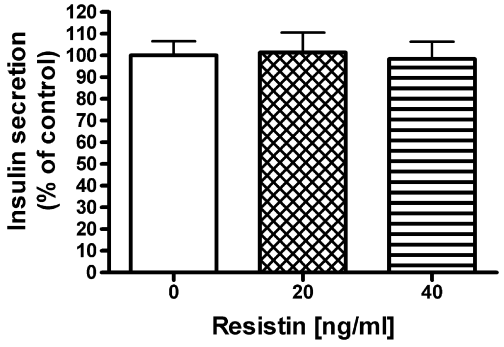

Fig. 4. Resistin has no effect on insulin secretion from BRIN-BD11 cells. Incubation with either 20 ng/ml or 40 ng/ml resistin for 24 h had no significant effect on subsequent 1 h GSIS (stimulated at 22 mM glucose) compared to control cells ($n = 4$ for each treatment).

5. Discussion

The potential role of resistin as a link between obesity and T2D in humans is controversial. This investigation however provides evidence that high concentrations of resistin could possibly lead to negative effects in pancreatic beta-cells, via a down-regulation of expression of the insulin receptor. We show here that when beta-cells are exposed to the kind of elevated levels of resistin which might be seen in obesity/T2D, a significant reduction in the level of insulin receptor mRNA and protein is seen, with decreases of 70% and 60%, respectively being seen. This observation is a novel one, as not only is there a dearth of published data involving resistin regulation of gene expression in the beta-cell, but there is also a lack of published material dealing with any effects that resistin might have on insulin receptor expression in non-islet tissue, even though data suggesting that resistin has a role in insulin resistance in rodents [8].

The observation that resistin can reduce insulin receptor expression in the beta-cell has profound importance. Research has shown that when the insulin receptor is knocked out in a beta-cell specific manner, hyperglycaemia results due to a loss of both beta-cell mass and secretory function [21], suggesting a central role for autocrine actions of insulin in maintaining pancreatic function. This finding suggests that if the effect that we report here occurs in vivo, then resistin may provide a link between increased adiposity and dysregulation of pancreatic beta-cell function and mass. Interestingly, the current study also found that incubation with this high concentration of resistin did not cause any significant change in the ability of the beta-cell to secrete insulin in response to high glucose challenge. This is in contrast to a recent study which found that at elevated glucose concentrations resistin caused a decrease in GSIS in isolated islet tissue (although this study used a much lower glucose concentration than was used here, and islet tissue is well established as being more 'glucose-responsive' than clonal beta-cell lines) [23]. Both these studies used 24-h incubations to test resistin's effects on GSIS. Whether or not longer-term exposure to elevated resistin levels directly regulates insulin secretion is a matter for future study, and might provide valuable information regarding the role of resistin in regulation of insulin secretion.

The novel observation that resistin regulates beta-cell viability is a particularly interesting one. Fig. 3 shows that at lower concentrations of resistin (similar to those reported in normal lean subjects) beta-cell viability was significantly increased by 50% compared to control cells. However, when the concentration of resistin was increased to levels which might be seen in obesity or T2D (30–40 ng/ml), this effect was reversed and there was no detectable change in cell viability compared to control cells. These data suggest the possibility that at the lower and more 'physiological' levels, resistin may act as a beta-cell growth factor (or possibly might combine this with an anti-apoptotic effect), potentially as part of an adipo-insular axis whose role it is to maintain beta-cell mass under 'adipotoxic' challenge. However at the much higher, and more 'pathological' resistin concentrations this proliferative effect does not occur, potentially suggesting that any axis of this type might become defective in the presence of elevated serum resistin concentrations. We have also observed in our lab that other adipokines can elicit a similar proliferative effect on clonal beta-cells and when this is taken into account with the well

established observation that beta-cell mass is decreased in T2D (reviewed in [22]) it becomes clear that this might be a possible mechanism by which increased adiposity negatively regulates beta-cell mass as well as beta-cell function.

It is also very interesting that resistin is expressed in the beta-cell, and at relatively high levels. The presence of resistin mRNA has previously been reported in human islets [15], and our results suggest that this expression occurs specifically in the beta-cell as well as in the whole islet. Expression levels were found to be high, with Ct values of between 23 and 26 being seen. Quite what the role of resistin in the beta-cell might be remains poorly understood, and is an area which could provide valuable information regarding the actions of this adipokine in the pathogenesis of T2D, although the possibility exists that it could act as a mediator.

The observations reported here are of particular importance in light of a recently published study, which showed that resistin has the ability to induce insulin resistance in pancreatic islets [23]. It is entirely possible that this reported induction of pancreatic islet insulin resistance by resistin is related to the decrease in insulin receptor expression that we report here; further studies will be required to confirm this. In conclusion our results suggest a central role for resistin in modulating beta-cell function in obesity and T2D, and therefore might allow future research to exploit this as a therapeutic avenue.

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